FEBS 19059 FEBS Letters 413 (1997) 185–190

Annexin I targets S100C to early endosomes

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Received 28 June 1997; revised version received 10 July 1997

Abstract Immunofluorescence and subcellular fractionation localize annexin I and the EF hand protein S100C to the same membranous structures which in part correspond to transferrin receptor-positive endosomes. The association of S100C with endosomal membranes is strictly dependent on annexin I binding since a D91stop–S100C mutant protein, in which the residues essential for annexin I binding have been removed, fails to co-localize with membraneous structures. This indicates that annexin I and S100C form a complex in vivo and that the endosomal localization of this complex is mediated through an interaction of annexin I with the endosomal membrane.

Key words: Annexin; Ca^{2+} binding protein; EF hand; Endocytosis

1. Introduction

Annexins comprise a multigene family of soluble proteins which interact in a Ca^{2+} dependent manner with phospholipids and intracellular membranes. Structurally, members of the family are characterized by repeats of a highly conserved 70–80 amino acid segment, the annexin repeat, which harbours a novel type of Ca^{2+} binding site [1]. Typically four of the annexin repeats form a highly conserved protein core domain. This core is preceded by a N-terminal domain unique in length and sequence within a given annexin protein [2]. The variable N-terminal domain is thought to determine the specific function of each annexin since it contains phosphorylation sites for different protein kinases and/or binding sites for protein ligands [3].

Annexins are present on most intracellular membranes and various members have been implicated in different membrane transport events [4,5]. These include the sorting of transport vesicles to the apical plasma membrane in MDCK cells which seems to depend on annexin XIIIb [6]. Annexin II; on the other hand, is a major component of early endosomes which are capable of homotypic fusion [7] and it regulates the distribution of early endosomes in vivo [8]. Furthermore, annexin II seems to play a major role in a Ca^{2+} dependent fusion process between early endosomes [9]. Another annexin possibly involved in endocytic processes is annexin I. It is a major substrate of the EGF receptor kinase and phosphorylation occurs in multivesicular bodies, endosomal structures implicated in the sorting of molecules destined for degradation to late endosomes/lysosomes [10,11]. Therefore annexin I has been linked to the inward vesiculation in multivesicular bodies and thus to the sorting of the EGF receptor to the degradative pathway [11]. Moreover, annexin I is specifically localized on early endosomes and might be involved in the structural organization of these membranes [12].

Conceptually, different intracellular activities of annexins could be specified through the unique N-terminal domains which could interact with regulatory protein ligands specific for individual annexins. Such annexin protein complexes exist and in three cases members of the annexin family have been shown to interact through their N-terminal domains with members of the S100 family of small dimeric EF hand type Ca^{2+} binding proteins [13]. Annexin XI binds S100A6 [14], annexin II forms a complex with p11 [15] and annexin I interacts with S100C [16,17]. The annexin II–p11 and annexin I–S100C complexes show a high structural similarity since the same regions of the respective proteins are involved in complex formation. However, only the annexin II–p11 complex has been characterized in vivo. A detailed analysis of the subcellular location and the characterization of the fate of ectopically expressed annexin II mutants revealed that annexin II–p11 complex formation is a prerequisite for anchoring annexin II in the cortical cytoskeleton of cultured cells [18,19].

While the structural requirements of the annexin I–S100C complex formation are well characterized in vitro [16,17], very little is known about the pysiological relevance of this interaction. Therefore we analyzed the annexin I–S100C complex formation within cells. By characterizing the intracellular distribution of annexin I, S100C and ectopically expressed S100C mutants impaired in annexin I binding we show that annexin I forms a complex with S100C in vivo and that this complex is localized on early endosomal membranes.

2. Materials and methods

2.1. Cell culture and transfection

LLCPK and BHK cells were grown in DME (Gibco-BRL) supplemented with 10% FCS (Sigma) at 37°C and 5% CO_2 in a humidified atmosphere. For transient transfections the wt–S100C cDNA [17] and the D91stop–S100C mutant cDNA (lacking residues 91–99 and thus the annexin I-binding site [17]) were cloned into the pCMV5 vector to yield the constructs pCMV S100C and pCMV D91stop S100C, respectively. The expression constructs encoding the human transferrin receptor (pCMV hTfR) and porcine annexin I (pCMV AnxI) have been described [8,12,20]. LLCPK cells were transiently transfected with pCMV hTfR using the LipofectAmin system (Gibco-BRL) according to the manufaturer's instructions. BHK cells were transiently transfected with one S100C expression construct (pCMV S100C, pCMV D91stop S100C) and pCMV AnxI or pCMV hTfR using the modified calcium phosphate precipitation method [21]. The BHK cells were transfected at 50% confluence in 100 mm dishes with 15 μg plasmid DNA. After incubation for 12–16 h at 35°C in 3% CO_2 the cells were washed with PBS and then cultivated in fresh medium employing normal culture conditions. Thirty-six to 40 h following transfection, 30–40% of the total cell population was expressing the exogenous proteins, as monitored by immunofluorescence using an antibody specifically recognizing the ectopically expressed proteins.

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PII S0014-5793(97)00911-3

2.2. Immunofluorescence

Following transfection of BHK cells grown on glass coverslips hTfR internalization into early and recycling endosomes was stimulated by incubating the transfected cells for 30 min in serum-free DME medium and then for 30 min in serum-free medium containing 20 μg/ml human transferrin (Boehringer Mannheim). Permeabilization and cytosol depletion of BHK cells was then achieved by treatment with streptolysin O (SLO; kindly provided by Dr. S. Bhakdi, University of Mainz, Germany) as described [22]. Briefly, the coverslips were washed with ice-cold PBS and SLO was bound to the plasma membrane by incubating the cells for 10 min on ice with 50 μl of preactivated SLO at a concentration of 5 μl/ml in intracellular transport buffer (ICT: 70 mM HEPES/KOH pH 7.0, 78 mM KCl, 4 mM $MgCl_2$, 10 mM EGTA, 8.37 mM $CaCl_2$, 1 mM DTT; [23]). Unbound SLO was removed by washing twice with ice-cold ICT. The cells were permeabilized by incubating the coverslips for 10 min at 37°C in ICT. Cytosol was further depleted by washing for 10 min in ice-cold ICT and the cells were then fixed for 2 min in −20°C cold methanol. LLCPK cells were fixed and permeabilized for 6 min in methanol at −20°C. The fixed LLCPK and BHK cells were washed with PBS and incubated with the first antibodies in PBS containing 1 mg/ml BSA for 60 min at 37°C. After washing in PBS, the cells were incubated with the secondary antibodies, washed again and mounted in Moviol 4-88 (Hoechst). Fluorescence analysis was performed using a Zeiss Axiophot photomicroscope and images were photographed using a Tri X Pan 400 film (Kodak).

2.3. Fractionation of endosomes

The fractionation of endosomes was carried out essentially as descibed [24,25]. Briefly, six 10 cm dishes of transfected BHK cells were treated with 2 mg/ml horseradish peroxidase (HRP) in internalization medium (IM: DME medium with 10 mM HEPES, pH 7.4) at 37°C. HRP was endocytosed for 5 min to label early endosomes. To label late endosomes, the cells were treated for 5 min with HRP and then the HRP was chased along the endocytic pathway by incubating the cells for 45 min in IM supplemented with 2 mg/ml BSA [24]. Subsequently, the cells were lysed in homogenization buffer (HB buffer: 250 mM sucrose, 3 mM imidazole/HCl, pH 7.4) containing protease inhibitors (10 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml antipain, 1 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, 0.5 mM benzamidine, 10 μM E-64) and the mixture was centrifuged to obtain a post-nuclear supernatant (PNS). The PNS was adjusted to 40.6% sucrose, 3 mM imidazole, pH 7.4, and placed at the bottom of a SW60 centrifugation tube (Beckman). This was overlaid with 1.5 ml of 35% sucrose, 3 mM imidazole/HCl, pH 7.4, then with 1 ml of 25% sucrose, 3 mM imidazole/HCl, pH 7.4, and finally with 0.5 ml of HB buffer. All sucrose solutions containded the same cocktail of protease inhibitors as the HB buffer. The step gradient was centrifuged at 35 000 rpm for 60 min at 4°C in a SW60 rotor. The 25% HB (membranes enriched in late endosomes/endosomal carrier vesicles), 25–35% (early endosomal membranes) and 35–40.6% (heavy membranes) sucrose interphases were collected and the activity of the endocytosed HRP present in the different fractions was analysed as described [26]. Latency was measured to verify the integrity of the isolated endosomes [27]. For analysis of the wt– and D91stop–S100C distribution in the gradient, proteins present in the individual fractions were concentrated by chlorofom/methanol precipitation [28] and 10 μg total protein of each fraction [29] were seperated by SDS-polyacryamide gel electrophoresis using the tricine buffer system [30]. The proteins were transferred by electrophoretic blotting (transfer buffer: 50 mM Tris, 50 mM boric acid) to polyvinyldifluoride membranes (ProBlott Membranes; Applied Biosystems) and analyzed by immunoblotting with a polyclonal S100C antibody [31].

2.4. Antibodies

Affinity purification of the rabbit polyclonal S100C antibody [17] was performed using purified recombinant S100C coupled to cyanogen bromide activated Sepharose (Pharmacia). The human transferrin receptor was detected with the monoclonal antibody (mAb) B3/25 (Boehringer Mannheim). The mouse monoclonal antibody against annexin I has been described [12]. For immunoblot analysis peroxidase coupled secondary antibodies (Dako) were used and immunoreactive bands were detected using the enhanced chemoluminescence system (Amersham). Double immunofluorescence employed rhodamine-conjugated goat anti-rabbit IgGs (Dianova) and FITC-coupled goat anti-mouse IgGs (Cappel Laboratories) as secondary antibodies.

3. Results and discussion

3.1. Intracellular localization of S100C in LLCPK cells

We first analyzed the intracellular distribution of S100C in LLCPK cells by immunofluorescence microscopy employing an affinity purified polyclonal antibody raised against recombinantly expressed S100C. The cells were fixed and permeabilized with methanol and the high cytosolic pool of S100C was depleted by washing with PBS. Labeling for S100C is apparent underneath the plasma membrane, in particular at sites of cell-to-cell contact and in small dots throughout the cytoplasm, which are concentrated in a distinct cluster in the perinuclear area (Fig. 1). Since this staining pattern appeared similar to the localization of the transferrin receptor (TfR), we compared the distribution of S100C directly to that of the TfR by double immunofluorescence. The transferrin receptor is a marker for early endosomes and accumulates during its recycling pathway in an endosomal compartment in the pericentriolar region of the cell [32,33]. As the endogenous expression level of the transferrin receptor in LLCPK cells was too low for localization studies, we employed a transient transfection approach to overexpress the transferin receptor. Therefore we used an expression plasmid encoding the human transferrin receptor (hTfR) which was previously shown to yield high expression levels in combination with a proper targeting of hTfR to the correct endosomal compartments [8,20]. About 38 h following transfection the cells were subjected to double immunofluorescence using antibodies against S100C and the ectopically expressed hTfR. Fig. 1 shows that a significant fraction of S100C co-localizes with the hTfR positive endosomal structures.

3.2. S100C is enriched in endosomal membrane fractions

To corroborate the localization of S100C on early/recycling endosomal membranes we analyzed subcellular membrane fractions of BHK cells for their S100C content. We used BHK cells for this analysis since a fractionation method enriching for endosomal membranes is well established for these cells [24,25]. Since our polyclonal S100C antibody did not react with the endogenous hamster (BHK) protein we chose a transient transfection approach to express in BHK cells porcine S100C which is recognized by the antibody. As monitored by immunofluorescence 40% of the total cell population expressed the exogenous protein 40 h following transfection (not shown). To label endosomes prior to the subcellular fractionation, the transfected cells were treated either with HRP for 5 min (fluid phase uptake into early endosomes) or with HRP for 5 min followed by a 45 min chase in marker-free medium (HRP transport to late endosomes). Subsequently the cells were harvested and cellular membranes were subjected to a step gradient fractionation to enrich for endosomal membranes [24,25]. The following immunoblot analysis of the different fractions revealed that S100C is indeed enriched in the fractions containing early endosomes (Fig. 2A).

Thus our morphological and biochemical analyses show that at least a large fraction of the membrane bound S100C is associated with early endosomes. This is reminiscent of the intracellular distribution of annexin I which was recently shown to form a complex with S100C in vitro [12,16,17]. Since

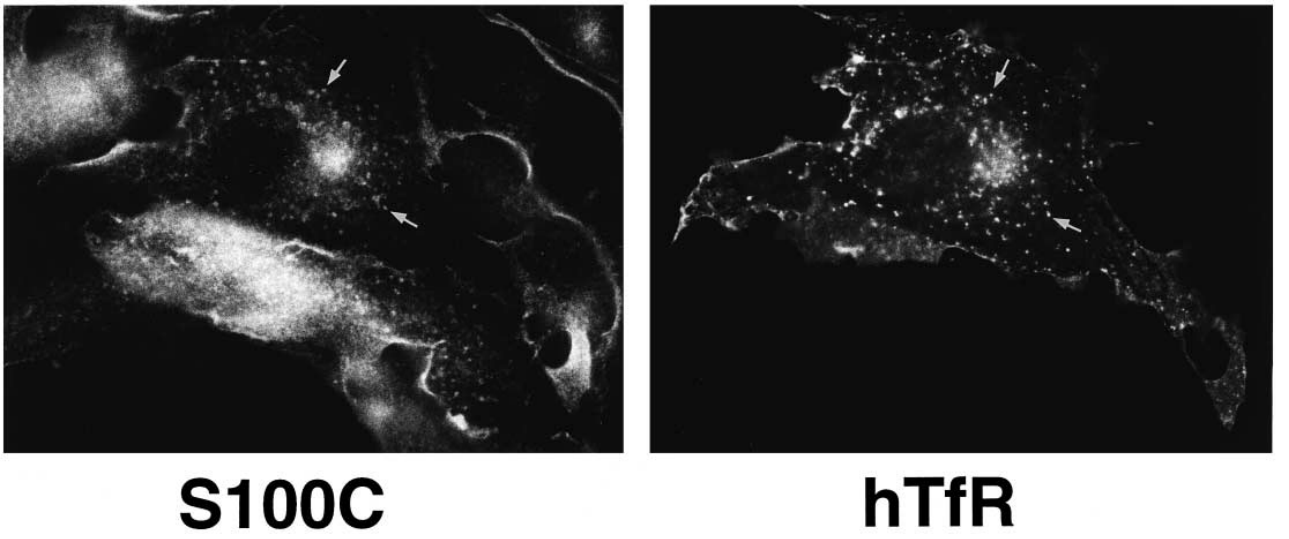

Fig. 1. Immunofluorescence analysis of the S100C localization in LLCPK cells. LLCPK cells ectopically expressing the human transferrin receptor (hTfR) were fixed and permeabilized by treatment with cold methanol. Cells were then double labeled with antibodies to S100C and hTfR and the distributions were visualized by labeling with the appropriate secondary antibodies coupled to rhodamine (S100C) and FITC (TfR) respectively.

the expression patterns of annexin I and S100C also show a high degree of similarity [34–36] it seems likely that the two proteins also form complex in vivo.

3.3. *Association of S100C with endosomes depends on the annexin I–S100C complex formation*

To determine directly whether S100C and annexin I form a complex within the cell we expressed in BHK cells a mutant S100C protein incapable of annexin I binding and analyzed the subcellular distribution of this mutant. We chose for this approach the D91stop–S100C mutant which has suffered a deletion of the hydrophobic C-terminal extension encompassing residues 91–99 and thus the binding site for annexin I [17]. To detect the mutant protein in transfected BHK cells we employed a polyclonal S100C antibody specifically recognizing the ectopically expressed and not the endogenous protein. As revealed by immunofluorescence, the mutant protein shows the same high expression rate and level as the transiently expressed wild-type (wt) protein (not shown). However, in contrast to transfected wt–S100C the mutant protein fails

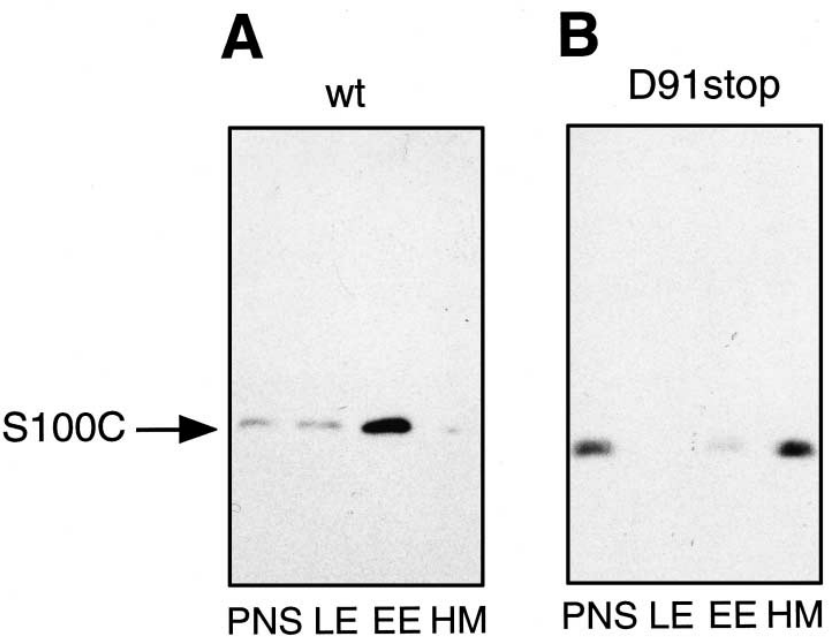

Fig. 2. Distribution of wt– and D91stop–S100C among subcellular fractions of BHK cells. A postnuclear supernatant (PNS) prepared from BHK cells ectopically expressing wt– or D91stop–S100C was fractionated on a discontinuous flotation gradient. Following centrifugation, fractions enriched in late endosomes/endosomal carrier vesicles (LE), early endosomes (EE) and heavy membranes (HM) were collected. 10 μg of total protein of each fraction were sujected to SDS-PAGE followed by immunoblot analysis with an antibody only recognizing the ectopically expressed S100C proteins. Note that wt– (A) but not D91stop–S100C (B) is enriched on early endosomal membranes.

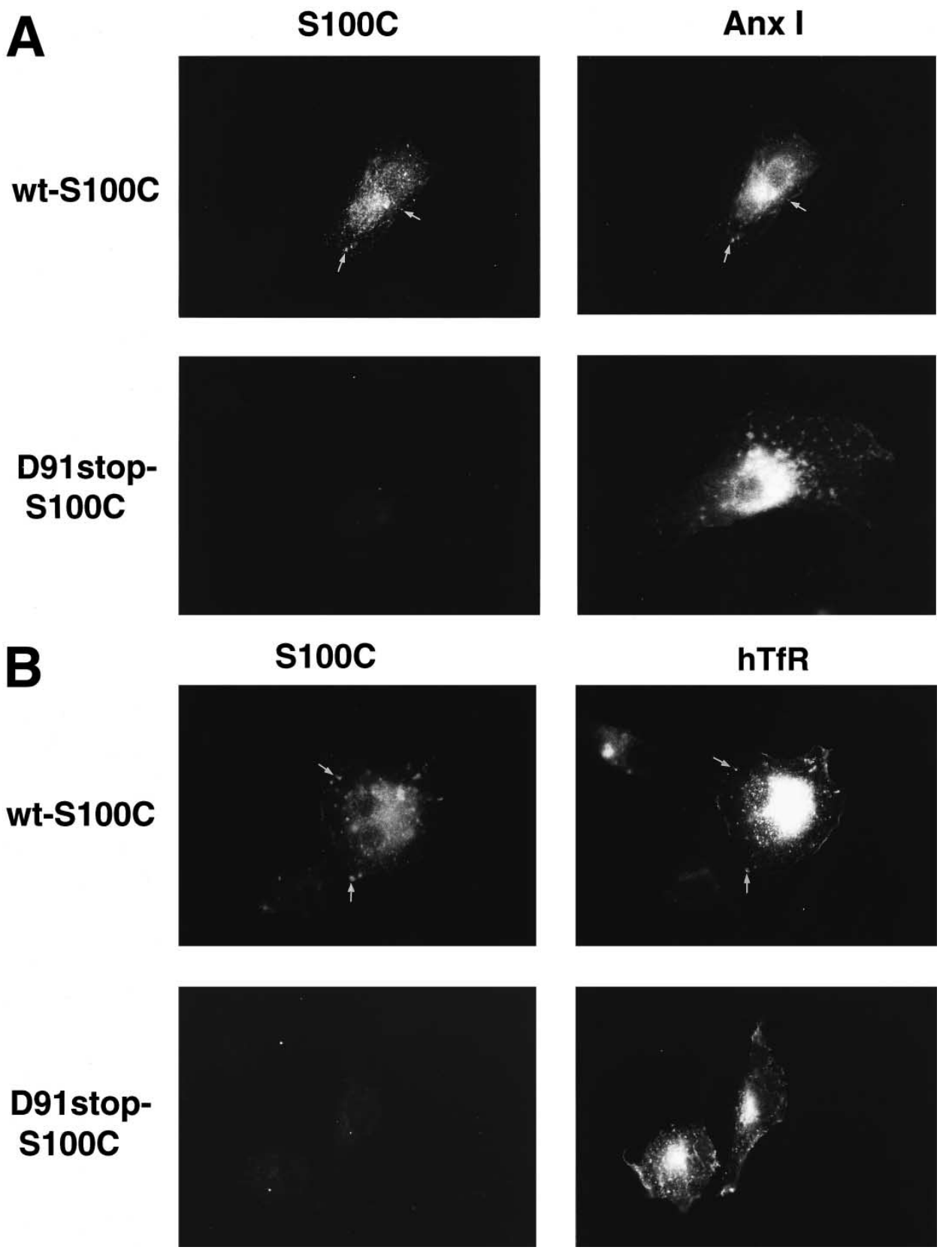

Fig. 3. Localization of wt– and D91stop–S100C in BHK cells. BHK cells were transiently co-transfected with constructs expressing wt– or D91stop–S100C and the human transferrin receptor (hTfR; upper panels) or annexin I (AnxI; lower panels), respectively. After permeablization and cytosol depletion by treatment with SLO the cells were fixed and double labeled with antibodies against S100C and annexin I or hTfR, respectively. All antibodies recognized only the ectopically expressed and not the endogenous proteins. Only wt– but not D91stop–S100C co-localizes with annexin I and transferrin receptor-positive endosomes. Arrows mark examples of such co-localizations.

to co-fractionate with endosomal membranes (Fig. 2). This is not due to possible changes in the endosomal membranes which may have resulted from the overexpression of the mutant S100C protein since there were no differences in the distribution and the quantity of the endocytosed marker HRP among the gradient fractions of D91stop– and wt–S100C expressing cells (not shown). These observations indicate that the association of S100C with early endosomal membrane fractions depends on annexin I binding and that an endosomally localized annexin I–S100C complex is formed in vivo.

To verify the results of the subcellular fractionation, we compared the localization of wt– and mutant–S100C in BHK cells by double immunofluorescence analyses. BHK cells were transiently co-transfected with plasmids coding for S100C and hTfR or those encoding S100C and annexin I, respectively. Since there is a high cytosolic pool of S100C in BHK cells, the cells were depleted of cytosol by streptolysin O permeabilization prior to fixation. This allowed a specific visualization of the membrane bound pool of S100C and annexin I. In a first set of experiments, we compared the distribution of wt and mutant S100C to that of the hTfR. Fig. 3 reveals a high degree of colocalization of wt–S100C with hTfR positive endosomal structures. This localization of the transfected S100C is essentially the same as that of endogenous S100C in LLCPK cells (see Fig. 1). However, in BHK cells the perinuclear concentration of the hTfR and the S100C staining is less evident and more dots are present. This is in line with earlier findings on the subcellular distribution of the recycling endosomes in BHK and CHO cells [37]. In contrast to wt–S100C, the D91stop–S100C mutant lacking the annexin I binding site does not co-localize with the TfR positive membranes indicating that annexin I binding is a prerequisite for the early endosomal association of S100C (Fig. 3). As a further control, we directly compared the distribution of S100C and annexin I. Fig. 3 shows that wt– but not D91stop–S100C colocalizes with annexin I. The D91stop–S100C mutant was removed during the cytosol depletion, whereas annexin I and wt–S100C remained membrane bound. This indicates that binding of S100C to membranes requires an interaction with annexin I thus corroborating our biochemical data on the interaction of annexin I and S100C in vivo.

Two other members of the annexin family have been shown to interact with S100 proteins. Annexin XI binds calcyclin [14] and annexin II forms a complex with p11 [15]. The annexin II–p11 and annexin I–S100C complexes share the same structural principles since the same regions in the respective proteins participate in the protein–protein interaction, i.e. in each case the unique N-terminal region of the annexin binds to the C-terminal extension of the S100 protein. However, the annexin I–S100C complex is regulated by Ca^{2+}, whereas p11 binds Ca^{2+} independetly to annexin II [15,17]. Binding of Ca^{2+} to S100C most likely exposes on the surface of the protein hydrophobic residues of the C-terminal extension which then interact with annexin I. The same C-terminal extension is also indispensable for the endosomal localization of S100C as the mutant lacking this domain fails to show any membrane association.

It seems likely that the formation of the annexin I–S100C complex modulates the physiological properties of annexin I. In vitro it has been shown that the binding of S100C suppresses the phosphorylation of annexin I by protein kinase C [38]. Phosphorylation by PKC occurs in the N-terminal domain of annexin I and changes the Ca^{2+} and phospholipid binding properties since the Ca^{2+} concentrations necessary for the annexin I dependent aggregation of phospholipid vesicles [39] and chromaffin granules [40] are increased. Such a regulation by PKC phosphorylation and S100C binding could affect the role annexin I has been proposed to play in the internal vesiculation process in multvesicular endosomes [11] and in the structural organization of early endosomes [12]. Since S100C forms a tight homodimer under physiological conditions, a following binding of each subunit to one annexin I molecule would result in the formation of a heterotetrameric complex. Provided that each annexin I molecule is associated with different endosomal membranes or different regions of an endosomal compartment the heterotetrameric complex could connect these membranes. This physical linkage could possibly hold the membranes in place for the actual fusion process to occur and/or could organize the endosomal compartment.

Acknowledgements: We thank Mary Osborn for helpful discussions. This work was supported in part by grants from the DFG and the HFSPO (to V.G.) and by a postdoctoral fellowship by the Max Planck society to J.S.

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